DISSERTATION ON

IMMUNE RESPONSE IN TUBERCULOSIS

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CERTIFICATE

This is to certify that this dissertation entitled **"IMMUNE RESPONSE IN TUBERCULOSIS"** submitted by **Dr.Umashankar.L** appearing for Part II M.D. Branch I General Medicine Degree examination in September 2006 is a bonafide record of work done by him under my direct audience and supervision in partial fulfillment of regulations of the Tamil Nadu Dr. M.G.R. Medical University, Chennai. I forward this to the Tamil Nadu Dr.M.G.R. Medical University, Chennai, Tamil Nadu, India

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CONTENTS

Introduction

Tuberculosis is a global emergency. One third of the world's population is infected, and although only about 5–10% develops active disease during the first few years following exposure [1], this still results in a massive case load, with eight million new cases each year, and three million deaths. Moreover, the percentage that progresses to disease is increasing. Tuberculosis is one of the first secondary infections to be activated in human immunodeficiency virus (HIV)-positive individuals [4].

Moreover the stresses of poverty, malnutrition and war, increase the rate of reactivation for reasons discussed later. Even in developed countries such as the United Kingdom, the disease distribution in large cities parallels the distribution of poverty [3]. Meanwhile the breakdown of healthcare systems is leading to incomplete case and contact tracing, incomplete treatment, and increases in drug resistance. In some parts of the world, many of the available drugs are fake or out of date [4]. In many areas, existing treatment is probably doing more harm than good, as incomplete treatment regimens select for drug resistance.

Multidrug-resistant tuberculosis is spreading at an alarming rate, especially in a developing country like our India. There were more cases of tuberculosis in 2005 than ever before in the history of mankind.

We need new insights that lead to more rapid therapies and immuno therapies, and more reliable vaccines. Recent insights have come from, understanding of the relationship between *Mycobacterium tuberculosis* and macrophages; the multiple T cell types that recognize mycobacterial peptides, lipids and glycolipids; the critical role of interferon γ and interleukin-12 (IL-12) in human mycobacterial infection revealed by genetically defective children; quantization of the presence and importance of Th2 lymphocyte activation in human tuberculosis; the role of local conversion of inactive cortisone to active cortisol in the lesions; the recognition that some effective prophylactic vaccines also work as immunotherapeutic whereas others do not. In the longer term the recent sequencing of the *M. tuberculosis* genome will lead to further advances.

Pulmonary tuberculosis is an important clinical problem as about one third of the world's population is infected with Mycobacterium tuberculosis and tuberculosis continues to be a leading infectious disease worldwide. The current vaccine Mycobacterium bovis bacilli Calmette & Guerin (BCG), has variable protective efficacy, ranging from 0 to 85% in different studies [2]. Owing to the

variable efficacy and threat of disease in an immuno compromised host, the use of a live vaccine, BCG, is still a controversial subject. In this context **the use of second generation anti-TB vaccines and alternative immunomodulatory approaches to treatment are urgently required.**

Also, understanding the cellular and molecular basis of the protective memory immune response against M. tuberculosis would assist in designing new vaccines. There is compelling clinical evidence that, in addition to innate virulence of tubercle bacillus itself, the host response to M. tuberculosis plays a major role in determining the clinical manifestations and the ultimate outcome of the patients who encounter this pathogen [4].

Understanding the components of the host response at a basic level is likely to lead to a better understanding of the pathogenesis of TB in humans and result in better and novel approaches to prevention and therapy of this disease.

Among the many clinical manifestations of tuberculosis, tuberculous pleuritis is of particular interest. Host defence against Tuberculosis involves infiltration of the peripheral blood mononuclear cells (PBMC) into pleural

space [5]. Patients with TB mount a resistant immune response to infection, reflected by resolution of pleuritis even without chemotherapy in most cases[7]. Thus TB provides a very good model to study immune response in vivo, as the immunological reactivity against M. tuberculosis is compartmentalised in pleural space [8].

It is known that the critical component of protective immunity against TB is T cell-mediated response characterised by secretion of cytokines, primarily by the CD4+ T cells [9].

Knowing the cellular and humoral responses at the site of infection could give us a better understanding of the immune responses involved therein. Inflammatory cells and pleural fluid (PF) can readily be obtained from the infection site and characterisation of PF derived cell population and cytokines released could help us to gain further insight into the immunomodulatory processes involved in this disease. Hence it is decided to study immune responses in TB and a comparison of the systemic and local immune responses in such patients.

OBJECTIVES OF THE STUDY

1. To study immune responses in Tuberculosis with respect to Th1 and Th2 immunity, so that in future **immunotherapy of Tuberculosis can be targeted.**

2. To compare the systemic and local immune responses in tuberculous pleural effusion patients.

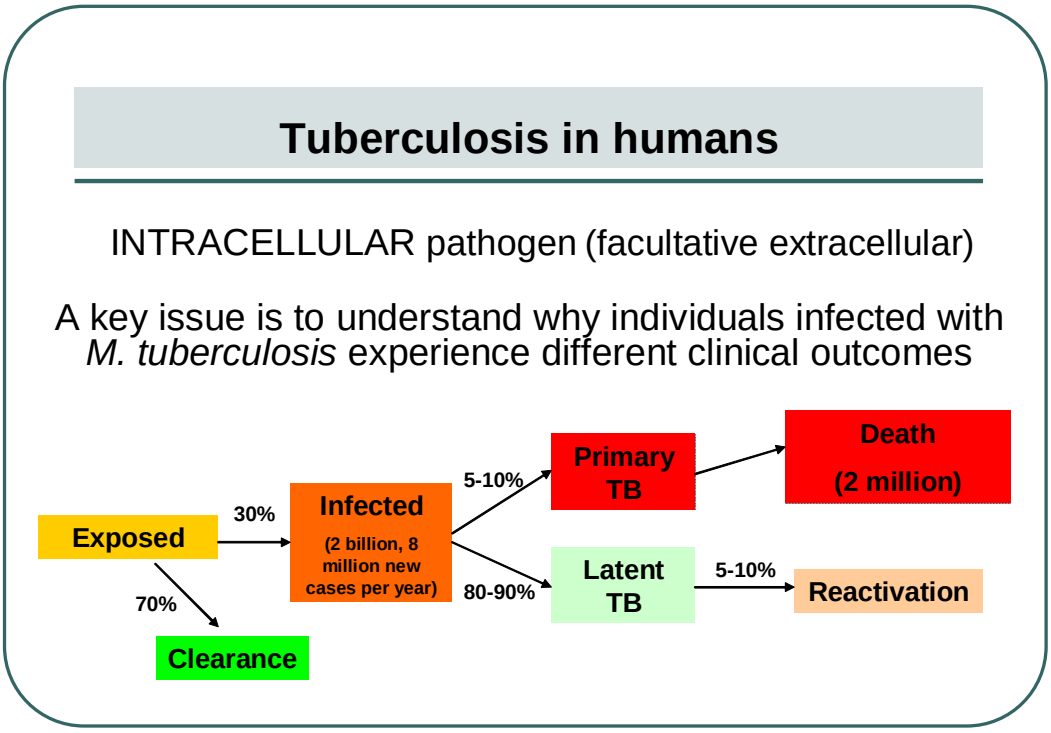
REVIEW OF LITERATURE

Incidence of tuberculosis is increasing. Current treatment regimens require at least 6 months, because latent or stationary phase organisms are difficult to kill. Such regimens do not achieve full compliance, and "directly observed therapy short course" (DOTS) is having less impact than expected.[23] This worrying situation is aggravated by co infection with human immunodeficiency virus (HIV), and by the increase in drug-resistant strains.

An important reason for the current failure to control tuberculosis is the fact that even the best available treatment must be continued for at least 6 months. This treatment regimen is not a realistic proposition in most developing countries, or even in the inner cities of rich ones, because the patients feel well after a few weeks and stop taking the drugs. The World Health Organization (WHO) now admits that directly observed therapy short-course (DOTS), in which the patient is supervised while taking every dose of therapy, helps but does not solve the problem [23].

Persistent bacilli and latent infection are two interrelated reasons for the requirement for 6 month regimens. The first is obvious and often discussed. The chemotherapy kills the vast majority of the bacteria within a few days, but there are subpopulations of "persisters" [23]. It is not clear whether these organisms are in true stationary phase [10] or merely replicating extremely slowly. Nor is it clear where they are located. Most authors assume that they are in old lesions or sites of fibrosis or calcification, where oxygen availability may be low. However, in a forgotten paper published in 1927, Opie and Aronson reported that 80% of tuberculous lesions were already sterile 5 yrs after the primary infection, whereas live bacilli could be found in macroscopically normal lung tissue. The fact that metronidazole, a drug that should be active under anaerobic conditions, is not active in a model of latent tuberculosis infection in mice, implies that live organisms also persist in well-oxygenated sites in this species [11].

Not only do persister organisms cause problems for treatment, but they also constitute an important source of infection. They can persist for the rest of the life of the individual [10], and, at least in countries with low or moderate tuberculosis endemicity, many cases of tuberculosis result from reactivation of latent infection[11].



Clinical Outcome of Tuberculosis

PATHOGENESIS

Mycobacterium tuberculosis is an obligatory aerobic, intracellular pathogen, which has a predilection for the lung tissue rich in oxygen supply. The tubercle bacilli enter the body via the respiratory route. The bacilli spread from the site of initial infection in the lung through the lymphatics or blood to other parts of the body, the apex of the lung and the regional lymph node being favoured sites. Extrapulmonary TB of the pleura, lymphatics, bone, genito-urinary system, meninges, peritoneum, or skin occurs in about 15 per cent of TB patients.

Events following entry of bacilli:

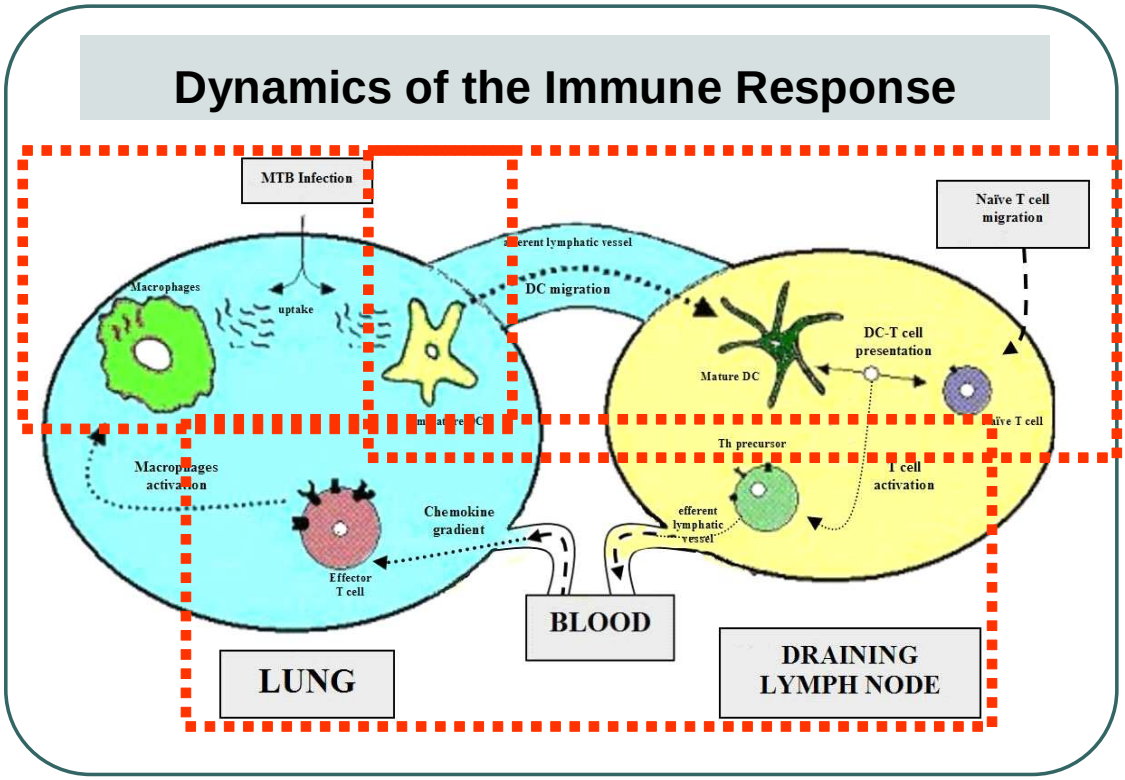
Phagocytosis of M.tuberculosis by alveolar macrophages is the first event in the host-pathogen relationship that decides outcome of infection. Within 2 to 6 wk of infection, cell-mediated immunity (CMI) develops, and there is an influx of lymphocytes and activated macrophages into the lesion resulting in granuloma formation. The exponential growth of the bacilli is checked and dead macrophages form a caseum. The bacilli are contained in the caseous centers of the granuloma. The bacilli may remain forever within the granuloma, get re-activated later or may get discharged into the airways after enormous increase in number, necrosis of bronchi and cavitations.

Fibrosis represents the last-ditch defense mechanism of the host, where it occurs surrounding a central area of necrosis to wall off the infection when all other mechanisms failed.

Macrophage-Mycobacterium interactions and the role of macrophage in host response can be summarized under the following headings:

1. Surface binding of M. tuberculosis to macrophages.
2. Phagosome-lysosome fusion.
3. Mycobacterial growth inhibition/killing.
4. Recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity.

When exudative pleural effusions accumulate in the pleural space, various cells produce many kinds of cytokines or chemokines that contribute to the progress of pleuritis [15]



Dynamics of Immune Response in Tuberculosis

On encountering an antigen, naïve CD4+ T-helper precursor cells enact a specific process that results in differentiation toward the T-helper type 1(Th1) or T-helper type 2 (Th2) lineages. Th1 cells produce interleukin (IL)-2 and interferon γ whereas Th2 cells produce IL-4, IL-5, and IL-10.

Th1 and Th2 dichotomy in TB:

Two broad (possibly overlapping) categories of T cells have been described, Th1 type and Th2 type, based on the pattern of cytokines they secrete, upon antigen stimulation.

Th1 cells secrete IL-2, IFN-γ and play a protective role in intracellular infections.

Th2 type cells secrete IL-4, IL-5 and IL-10 and are either irrelevant or exert a negative influence on the immune response.

The balance between the two types of response is reflected in the resultant host resistance against infection. The type of Th0 cells shows an intermediate cytokine secretion pattern. The differentiation of Th1 and Th2 from these precursor cells may be under the control of cytokines such as IL-12.

In mice infected with virulent strain of M. tuberculosis, initially Th1 like and later Th2 like response has been demonstrated [18]. There are inconsistent reports in literature on preponderance of Th1 type of cytokines, of Th2 type, increase of both, decrease of Th1, but not increase of Th2 etc.

Moreover, the response seems to vary between peripheral blood and site of lesion; among the different stages of the disease depending on the severity.

It has been reported that PBMC from TB patients, when stimulated in vitro with PPD, release lower levels of IFN-γ and IL-2, as compared to tuberculin positive healthy subjects [13]. Other studies have also reported reduced IFN-γ [14] increased IL-4 secretion [14] or increased number of IL-4 secreting cells [15]. These studies concluded that patients with TB had a Th2type response in their peripheral blood, whereas tuberculin positive patients had a Th1-type response.

More recently, cellular response at the actual sites of disease has been examined. Zhang et al studied cytokine production in pleural fluid and found high levels of IL-12 after stimulation of pleural fluid cells with M. tuberculosis. IL-12 is known to induce a Th1-type response in undifferentiated CD4+ cells and hence there is a Th1 response at the actual site of disease. The same group [17] observed that TB patients showed evidence of high IFNγ production and no IL4 secretion by the lymphocytes in the lymph nodes.

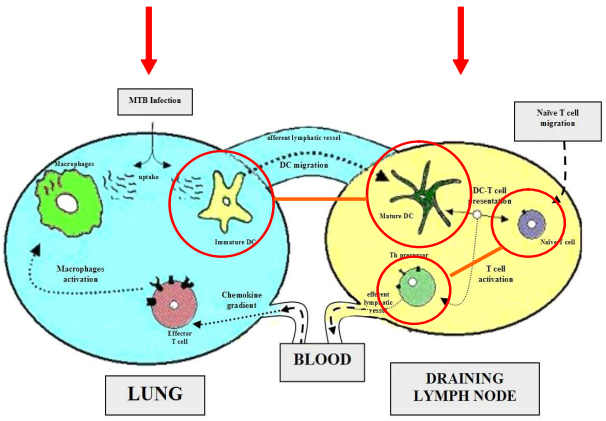
There was no enhancement of Th2 responses at the site of disease in human TB. Robinson et al [18] found increased levels of IFN-γ mRNA in situ in BAL cells from patients with active pulmonary TB.

In addition, reports suggest that in humans with TB, the strength of the Th1-type immune response relate directly to the clinical manifestations of the disease. Sodhi et al [19] have demonstrated that low levels of circulating IFN-γ in peripheral blood were associated with severe clinical TB. Patients with limited TB have an alveolar lymphocytosis in infected regions of the lung and these lymphocytes produce high levels of IFN-γ [20]. In patients with far advanced or cavitary disease, no Th1-type lymphocytosis is present.

New 2-compartmental model: LUNG + LN

Compartments
Lung (**L**) and draining Lymph Node (**LN**)

New variables
Dendritic Cells
IDC (immature, Lung)
MDC (mature, LN)
Lymphocytes (in the LN)
ThP (Precursor Helper Ts)
T (Naïve T cells)



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Human Immune Response to Mycobacterium tuberculosis

Cytokines

Interferon-γ:

IFN-γ, a key cytokine in control of M. tuberculosis infection is produced by both CD4+ and CD8+ T cells, as well as by NK cells. IFN-γ might augment antigen presentation, leading to recruitment of CD4+ T-lymphocytes and/or cytotoxic T-lymphocytes, which might participate in mycobacterial killing. Although IFN-γ production alone is insufficient to control M. tuberculosis infection, it is required for the protective response to this pathogen. IFN-γ is the major activator of macrophages and it causes mouse but not human macrophages to inhibit the growth of M. tuberculosis in vitro [19]. IL-4, IL-6 and GM-CSF could bring about in vitro killing of mycobacteria by macrophages either alone or in synergy with IFN-γ in the murine system1 [17]. IFN-γ GKO mice are most susceptible to virulent M. tuberculosis [21].

Humans defective in genes for IFN-γ or the IFN γ receptor are prone to serious mycobacterial infections, including M. tuberculosis [19].

Although IFN-γ production may vary among subjects, some studies suggest that IFN-γ levels are depressed in patients with active TB [20]. Another study demonstrated that M. tuberculosis could prevent macrophages from responding adequately to IFN-γ [21]. This suggests that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine.

One study comparing the immune response to pre-and post- BCG vaccination, has shown that BCG had little effect in driving the immune response towards IFN-γ and a protective Th1 response [18]. In another study on tuberculous pleuritis, a condition which may resolve without therapy, a protective Th1 type of response with increased IFN-γ is seen at the site of lesion (pleural fluid), while a Th0 type of response with both IFN-γ and IL-4 is seen under in vitro conditions [18].

To determine if the manifestations of initial infection with M. tuberculosis reflect changes in the balance of T cell cytokines, it was evaluated in vitro cytokine production of children with TB and healthy tuberculin reactors [24]. FN-γ production was most severely depressed in patients with moderately

advanced and far advanced pulmonary disease and in malnourished patients. Production of IL-12, IL-4 and IL-10 was similar in TB patients and healthy tuberculin reactors. These results indicate that the initial immune response to M. tuberculosis is associated with diminished IFN-γ production, which is not due to reduced production of IL-12 or enhanced production of IL-4 or IL-10.

Tumor necrosis factor (TNF-α):

TNF-α is believed to play multiple roles in immune and pathologic responses in TB. M. tuberculosis induces TNF-α secretion by macrophages, dendritic cells and T cells. In mice deficient in TNF–α or the TNF receptor, M. tuberculosis infection resulted in rapid death of the mice, with substantially higher bacterial burdens compared to control mice. TNF-α in synergy with IFN-γ induces NOS2 expression [25].

TNF-α is important for walling off infection and preventing dissemination. Convincing data on the importance of this cytokine in granuloma formation in TB and other mycobacterial diseases has been reported [21]. TNF-α affects cell migration and localization within tissues in M. tuberculosis infection. TNF-α influence expression of adhesion molecules as well as

chemokines and chemokines receptors, and this is certain to affect the formation of functional granuloma in infected tissues.

TNF-α has also been implicated in immuno pathologic response and is often a major factor in host-mediated destruction of lung tissue [27]. In one study, increased level of TNF-α was found at the site of lesion (pleural fluid), as compared to systemic response (blood) showing that the compartmentalized immune response must be containing the infection [8]

Interleukin-1:

IL-1, along with TNF-α, plays an important role in the acute phase response such as fever and cachexia, prominent in TB. In addition, IL-1 facilitates T lymphocyte expression of IL-2 receptors and IL-2 release. The major antigens of mycobacteria triggering IL-1 release and TNF-α have been identified [29]. IL-1 has been implicated in immunosuppressive mechanisms which is an important feature in immunity of tuberculosis [30]

Interleukin-2:

IL-2 has a pivotal role in generating an immune response by inducing an expansion of the pool of lymphocytes specific for an antigen. Therefore, IL-2 secretion by the protective CD4 Th1 cells is an important parameter to be measured and several studies have demonstrated that IL-2 can influence the course of mycobacterial infections, either alone or in combination with other cytokines [27].

Interleukin-4:

Th2 responses and IL-4 in TB are subjects of some controversy. In human studies, a depressed Th1 response, but not an enhanced Th2 response was observed in PBMC from TB patients. Elevated IFN-γ expression was detected in granuloma within lymph nodes of patients with tuberculous lymphadenitis, but little IL-4 mRNA was detected [31]. These results indicated that in humans a strong Th2 response is not associated with TB. Data from mice studies suggest that the absence of a Th1 response to M. tuberculosis does not necessarily promote a Th2 response and an IFN γ deficiency, rather than the presence of IL-4 or other Th2 cytokines, prevents control of infection. In a study of cytokine gene expression in the granuloma of patients with advanced

TB by in situ hybridization, IL-4 was detected in 3 of 5 patients, but never in the absence of IFN-γ expression [72]. The presence or absence of IL-4 did not correlate with improved clinical outcome or differences in granuloma stages or pathology.

Interleukin-6:

IL-6 has also been implicated in the host response to M. tuberculosis. This cytokine has multiple roles in the immune response, including inflammation, hematopoiesis and differentiation of T cells. A potential role for IL-6 in suppression of T cell responses was reported [33]. Early increase in lung burden in IL-6 -/-mice suggests that IL-6 is important in the initial innate response to the pathogen [37].

Interleukin-10:

IL-10 is considered to be an anti inflammatory cytokine. This cytokine, produced by macrophages and T cells during M. tuberculosis infection, possesses macrophage-deactivating properties, including down regulation of IL-12 production, which in turn decreases IFN-γ production by T cells. IL-10 directly inhibits CD4+ T cell responses, as well as by inhibiting APC functions

of cells infected with mycobacteria1 species [33]. Transgenic mice constitutively expressing IL-10 were less capable of clearing a BCG infection, although T cell responses including IFN-γ production were unimpaired [34]. These results suggested that IL-10 might counter the macrophage activating properties of IFN-γ.

Interleukin-12:

IL-12 is induced following phagocytosis of M. tuberculosis bacilli by macrophages and dendritic cells, which leads to development of a Th1 response with production of IFN-γ. IL-12p40-gene deficient mice were susceptible to infection and had increased bacterial burden, and decreased survival time, probably due to reduced IFNγ production [37]. Humans with mutations in IL-12p40 or the IL-12R genes present with reduced IFN-γ production from T cells and are more susceptible to disseminated BCG and M. avium infections [35]. An intriguing study indicated that administration of IL-12 DNA could substantially reduce bacterial numbers in mice with a chronic M. tuberculosis infection [36], suggesting that induction of this cytokine is an important factor in the design of a TB vaccine.

McDyer et al found that stimulated PBMC from MDR-TB patients had less secretion of IL-2 and IFNγ than did cells from healthy control subjects. IFNγ production could be restored if PBMC were supplemented with IL-12 prior to stimulation and antibodies to IL-12 caused a further decrease in IFNγ upon stimulation. Taha et al demonstrated that in patients with drug susceptible active TB both IFN-γ and IL-12 producing BAL cells were abundant as compared with BAL cells from patients with inactive TB.

Cell Mediated Immunity

So, tuberculosis is a granulomatous disorder, and the cellular immune response plays an important role in the defense mechanism. CD4 T-cells in tuberculous pleural effusions are Th1 dominant and are activated enough to produce Th1 cytokines. It was recently reported that the concentrations of IFN γ are all higher in tuberculous pleural effusions than in malignant pleural effusions IL-12 is known to induce a Th1 response in undifferentiated CD4+ cells, and this supports Th1 dominance at the morbid site of tuberculosis [36]. In Mycobacterium tuberculosis infections, IL-18 is also produced by activated macrophages and induces IFN-γ in synergy with IL-12cytokine status and T-

cell reactivity within the tuberculous pleural effusions were polarized strongly toward a Th1 response

The ability to manipulate the immune system of mice with neutralizing antibodies or gene knockout has provided strong evidence that in this species, immunity to tuberculosis correlates with a Type 1 response. *In vivo* T-helper (Th) 1 or Th2 cells act in concert with CD8 cells, and with numerous other cell types including macrophages, B cells and some stromal cells. Collectively these give rise to two patterns of cytokine release known as Type 1 (dominated by interleukin-2 (IL-2), interleukin-12 (IL-12), and interferon γ and Type 2 (dominated by interleukin-(IL)-4, 5, and 13) [14, 15]

Disruption of the major histocompatibility complex (MHC) Class II genes or of the gene for the ß chain of the T cell receptor resulting in a deficiency of $CD4^+$ T cells, render mice susceptible even to the avirulent Bacillus Calmette Guerin (BCG). Disruption of the gene for IFN γ makes mice very susceptible to *M. tuberculosis* (death within 3 weeks), and such mice may even die after many weeks from challenge with BCG. IL-18 knockout (KO)

mice are also more susceptible, perhaps because IL-18 contributes to the induction of IFN γ expression.

A major inducer of the Type 1 pathway is IL-12. The exact role of this cytokine depends on the mouse strain , but IL-12 KO mice are more susceptible to tuberculosis [16]

The detrimental role of Type 2 responses

Data emphasizes the crucial role of the Type 1 response. In agreement with this, other data indicate that the Type 2 response is not only unable to protect mice, but can seriously undermine the efficacy of the Type 1 response. If a weak Type 2 response to the shared mycobacterial antigens is deliberately induced before challenge, mice are found to be strikingly more susceptible to tuberculosis than are nonimmunised control animals [39].

Similarly, in the Balb/c mouse model of pulmonary (tuberculosis) TB infection, the appearance of IL-4 in the lung lesions (as seen by immuno

histochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) coincides temporally and spatially with the appearance of areas of pneumonia and necrosis, leading to rapid clinical deterioration and death. These observations are not contradicted by the claim that, in IL-4 gene-disrupted mice, there is no evidence of increased resistance to the infection . First, such mice are not devoid of Type 2 cytokine activity because IL-13 can substitute for many functions of the knocked out gene. Secondly, the detrimental role of the Type 2 response is most apparent in the late progressive phase of the disease, particularly after day 60

Type 2 responses in human tuberculosis

These observations, and above all, the susceptibility of children with defective receptors for IFN γ or IL-12, provide definitive evidence of the importance of Type 1 cytokines, and suggest a close parallel with the mouse models. Recently, the negative role of Type 2 cytokines in human tuberculosis (TB), again paralleling the murine models, has been established [39]. Expression of IL-4, whether measured by flow cytometry, or by sensitive quantitative RT-PCR on unstimulated peripheral blood T cells [40], is increased and correlates

with severity of disease and with cavitation. The IL-4 mRNA copy number also correlates with total immunoglobulin-E (Ig E) and with levels of soluble CD30..

Thus although it is true that actual cytokine levels and mRNA copy numbers are higher for Th1 than for Th2 cytokines in tuberculosis, the major change in cytokine expression compared to healthy donors is not as previously stated, the small decrease in expression of Th1 cytokines, but rather a massive (80–100-fold) increase in expression of Th2 cytokines [15] . This has resolved a long-running controversy which deserves explanation. That there is a Th2 component in the response of human tuberculosis patients to *M. tuberculosis* ought to have been accepted 10 years ago, because there is no other known explanation for the presence of specific IgE antibody [27].

Interestingly, the other largely Type 2 cytokine-dependent antibody, immunoglobulin-G4 (IgG4) is also reported to be increased in patients. Similarly, immunohistochemistry reveals IL-4-expressing cells in the lymphoid tissue of tuberculosis patients (but not in tissue from patients with sarcoidosis).

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PLEURAL EFFUSION

Pleural effusions, or abnormal accumulations of fluid in the pleural space, can be caused by a wide variety of intra thoracic and systemic diseases. In some cases, the etiology of a pleural effusion is apparent from the clinical context (eg, bilateral pleural effusion in congestive heart failure). However, the causes and clinical significance of the effusions often are not obvious. In most of these patients, a definitive or presumptive identification of the cause can be determined through analysis of pleural fluid obtained through thoracentesis.

Transudates are ultra filtrates of plasma in the pleura that arise from a small, defined group of causes. In contrast, exudates are produced by a variety of inflammatory conditions and often require more extensive evaluation and treatment

Pathophysiology

The inner surface of the chest wall and the surface of the lungs are covered by the parietal and visceral pleural, respectively, with a potential space of 10-24 µm between the 2 pleural surfaces. Normally, this space is filled with a

very small amount of fluid. However, large amounts of fluid can accumulate in the pleural space under pathological conditions.

The parietal pleura have sensory innervation and small apertures that aid in the absorption of particles and fluid. Both pleural surfaces mainly are supplied by systemic arterial vessels. Lymphatic vessels from the parietal pleura drain to lymph nodes along the anterior and posterior chest wall, while lymphatics from the visceral surface drain to the mediastinal lymph nodes. Normally, the pleural space contains a small amount of a colorless alkaline fluid (0.1-0.2 mL/kg), which has a low amount of protein (<1.5 g/dL). Approximately 90% of accumulated fluid in the pleural space is drained by the venous side, while the other 10% is absorbed by the lymphatics.

A delicate balance between the oncotic and hydrostatic pressures of the pleural space and the capillary intravascular compartments regulates filtration and drainage of pleural fluid. Hydrostatic and oncotic pressures are many-fold higher in the plasma than in the pleural space, but the net absorption of pleural fluid is slightly higher than the net filtration forces. In addition, the lymphatic drainage from the parietal pleura can surpass the rate of fluid filtration in the pleural space by several folds.

Chest wall and diaphragmatic movements enhance absorption of pleural fluid by the vascular and lymphatic vessels. Excessive filtration of fluid can overwhelm these efficient absorptive mechanisms and lead to the formation of pleural effusion.

Pleural effusions usually are classified as transudates and exudates. Diseases that affect the filtration of pleural fluid result in transudates formation, such as in congestive heart failure and nephritis. Transudates usually occur bilaterally because of the systemic nature of the causative disorders. Inflammation or injury increases pleural membrane permeability to proteins and various types of cells and leads to the formation of exudative effusion.

Mycobacterium tuberculosis invades the pleural cavity chiefly through rupture of sub pleural caseous foci within 6 to 12 weeks after a primary infection. Bacillus protein antigens seem to induce a delayed hypersensitivity reaction that stimulates lymphocytes, which in turn release certain lymphokines that (1) activate macrophages against the mycobacterium and (2) alter the permeability of pleural vessels and affect the formation of granuloma. Tuberculous pleural effusion (TPE) is an acute granulomatous pleurisy caused by recent infection by the mycobacterium. Patients with TPE invariably have a

small sub pleural nidus of tuberculosis showing fibrous and granulomatous inflammation and clear signs of leakage into the pleural space. Although TPE can resolve spontaneously within a few weeks or months, about one third of persons with untreated TPE subsequently develop a more serious form of tuberculosis.

Tuberculous pleural effusions are not always easy to diagnose because the standard criterion (the presence of a lymphocyte-rich exudates associated with caseous necrotic granuloma in pleural biopsy tissue samples, positive Ziehl-Neelsen stains or Lowenstein cultures of effusion or tissue samples, and cutaneous sensitivity to purified tuberculin protein antigen derivative [PPD]) is not invariably satisfied [6].

In recent years, numerous authors studied possible biochemical markers such as adenosine deaminase (ADA), ADA isoenzymes, lysozyme, interferon gamma, and other lymphokines to improve diagnostic efficiency, but diagnosis is still sometimes difficult.

Materials and methods

Study subjects:

Blood and PF were collected from 51 patients from our wards before the start of the treatment. The mean age of the study subjects was 38 years (range 18- 65 years). These patients were seronegative for human immunodeficiency virus (HIV).The blood and the PF samples collected for diagnostic and therapeutic purposes were utilised for the study. A written and informed consent was obtained from each patient. The collection of the samples and the study followed the ethical guidelines of Government General Hospital, Chennai.

Pleural effusions were defined as exudative using Light's criteria.

Light's criteria

The pleural fluid is an exudate if one or more of the following criteria are met:

- Pleural fluid protein divided by serum protein >0.5
- Pleural fluid LDH divided by serum LDH >0.6
- Pleural fluid LDH more than two-thirds the upper limits of normal serum LDH

Of the total, 51 patients were diagnosed to have tuberculosis based on the

1. Smear for AFB

2. Culture of the fluid (BACTEC)

3. Polymerase chain reaction (PCR) positivity (IS6110 specific) of the sputum or the PF together with the clinical picture.

These 31 patients showed positivity in at least any two of the above criteria and hence were categorised as TB group.

Non TB group

The remaining 20 patients, belonging to the Non TB group had exudative effusion with lymphocytic predominance due to causes other than tuberculosis. These patients had

1. Malignancy (N = 13)

2. Parapneumonic effusion (N = 4)

3. Exudative effusion due to secondary infections in SLE (N = 3)

All infectious but non-tuberculous pleural effusion was culture proven. The diagnosis of malignancy was on the basis of histology and cytology. Other cases were diagnosed based on compatible clinical and radiological findings. The diagnosis was also retrospectively proven as the disease was cured following the appropriate therapy.

In vivo and in vitro cytokine assay

Plasma (BL) and PF obtained from 51 patients were processed within 30min and were stored at 37 degree C, for measuring the in vivo cytokine levels. For in vitro cytokines, the PBMC and PFMC of the same 32 patients, at the concentration of 0.5U106 cells ml31 were cultured in 48-well plates (Costar, Cambridge, MA, USA) with antigens (PPD, CF, MTB, 10 Wgml31) or without antigens that served as control. The supernatants were collected after 48 h, when the maximum cytokine levels were found in initial standardization experiments. The cell-free supernatants were stored at 37 degree C for the cytokine assessment.

BL, PF and the supernatants of PBMC and PFMC were thawed at the time of cytokine enzyme-linked immunosorbent assay (ELISA). Measurement of interferon (IFN)-gamma, tumor necrosis factor (TNF) alpha, interleukin (IL)-12 p40 and IL-4 were done using the sandwich ELISA kit, according to the manufacturer's instruction (RpD Systems). The average of the duplicate readings was taken as the final concentration.

Cytokines (Lung)

IFN-γ production by other souces (NK, CD8,...), induced by IL-12 and B_T

IFN-γ production by Th1, induced by M_A

death

$$\frac{dI_\gamma}{dt} = s_g\left(\frac{B_T}{B_T + c_{10}}\right)\left(\frac{I_{12}^L}{I_{12}^L + sc_4}\right) + \alpha_5 T_1\left(\frac{M_A}{M_A + c_5}\right) - \mu_g I_\gamma \qquad (1.4)$$

IL-12 production by M_A and M_R

death

$$\frac{dI_{12}^L}{dt} = \alpha_8 M_A + \alpha_{23} M_R - \mu_{I_{12}^L} I_{12}^L \qquad (1.5)$$

IL-10 production by M_A, opposed by IFN-g and IL-10

IL-10 production by Th1, Th2 and ThP

IL-10 production by M_I

death

$$\frac{dI_{10}}{dt} = \alpha_{14} M_A\left(\frac{sc_6}{I_{10} + f_6 I_\gamma + sc_6}\right) + \alpha_{16} T_1 + \alpha_{17} T_2 + \alpha_{18} T_P^L + \delta_7 M_I - \mu_{I_{10}} I_{10} \qquad (1.6)$$

IL-4 production by ThP and Th2

death

$$\frac{dI_4}{dt} = \alpha_{11} T_P^L + \alpha_{12} T_2 - \mu_{I_4} I_4 \qquad (1.7)$$

Formulae Used for Measurement of Cytokines

Isolation of cells

PBMC and the PF mononuclear cells (PFMC) were isolated by Ficoll Hypaque (Amersham Pharmacia) density gradient centrifugation. The cells were washed twice in 1U Hank's balanced salt solution (HBSS) (Whittaker). A final re suspension was made in RPMI-1640(Sigma Chemicals Co.), supplemented with 10% autologous sera, to the required concentration.

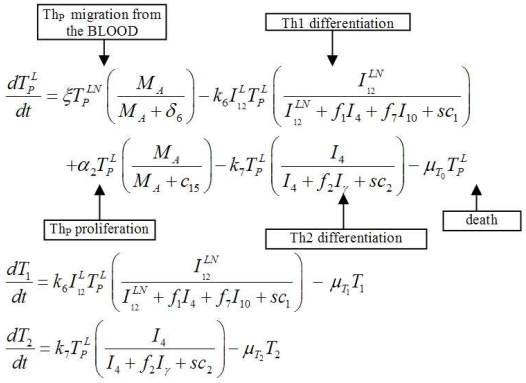
Flow cytometry analysis

We studied the percentage of various cell populations in PBMC and PFMC of 49 patients (31 TB and 18 NTB) by flow cytometric analysis. 100µl of the cell suspension containing 105 cells was washed with phosphate-buffered saline (PBS). A five-tube panel consisting of unstained cells, isotype control (mouse IgG conjugated to fluorescein thiocyanate (FITC) and Phycoerythrin (PE)), T cells/B cells marker (CD3 FITC/CD19 PE), helper T cells/cytotoxic T cells marker (CD4 FITC/CD8 PE), T cells/natural killer (NK) cells marker (CD3 FITC/CD16+56 PE), respectively were chosen. The cells were incubated with these monoclonal antibodies for 30min at 4‡C. Cell samples were washed twice with PBS and washed in 1% (w/v) paraformaldehyde. Cells were

acquired within 24 h, using the fluorescence-activated cell scan FAC Scan (Becton Dickinson)and the analyses were done using Cell Quest software.

The data were expressed as percentage of total lymphocytes. A total of 10 000 events for each sample were acquired. Fluorescence compensation on the flow cytometry was adjusted to minimize the overlap of the FITC and PE signals.

T helper cells (Lung)



$$\frac{dT_P^L}{dt} = \xi T_P^{LN}\left(\frac{M_A}{M_A+\delta_6}\right) - k_6 I_{12}^L T_P^L\left(\frac{I_{12}^{LN}}{I_{12}^{LN}+f_1 I_4+f_7 I_{10}+sc_1}\right) + \alpha_2 T_P^L\left(\frac{M_A}{M_A+c_{15}}\right) - k_7 T_P^L\left(\frac{I_4}{I_4+f_2 I_\gamma+sc_2}\right) - \mu_{T_0} T_P^L \quad (1.8)$$

$$\frac{dT_1}{dt} = k_6 I_{12}^L T_P^L\left(\frac{I_{12}^{LN}}{I_{12}^{LN}+f_1 I_4+f_7 I_{10}+sc_1}\right) - \mu_{T_1} T_1 \quad (1.9)$$

$$\frac{dT_2}{dt} = k_7 T_P^L\left(\frac{I_4}{I_4+f_2 I_\gamma+sc_2}\right) - \mu_{T_2} T_2 \quad (1.10)$$

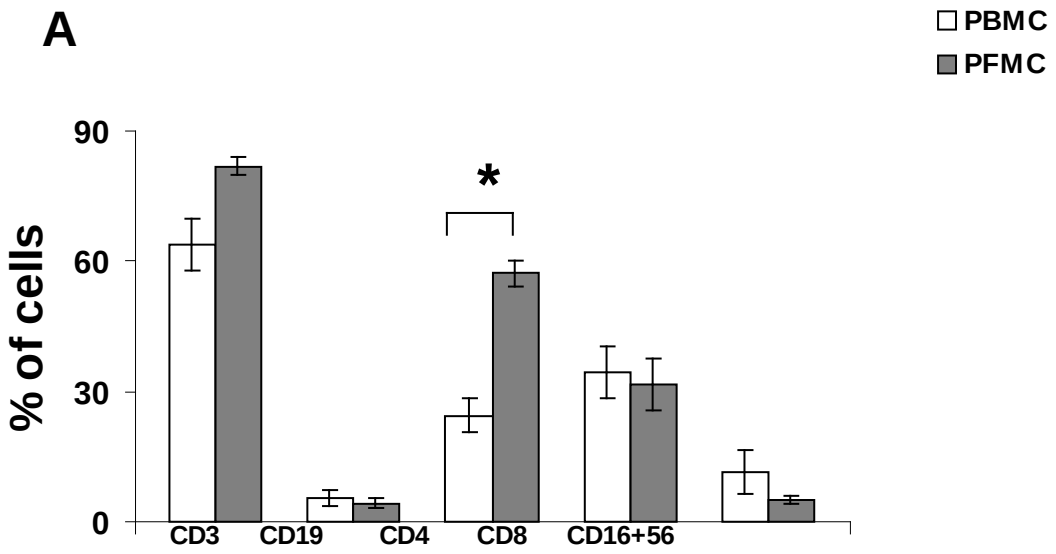
Formulae Used for calculation of T cells

Statistical analysis

Data are presented as the mean and S.E.M. in both text and in figures. For normally distributed data, comparisons between groups were done with the paired or unpaired Student's t-test, as appropriate. For data that were not normally distributed, the Wilcoxon rank sum test was used. $P < 0.05$ was considered to be statistically significant.

Results and discussion

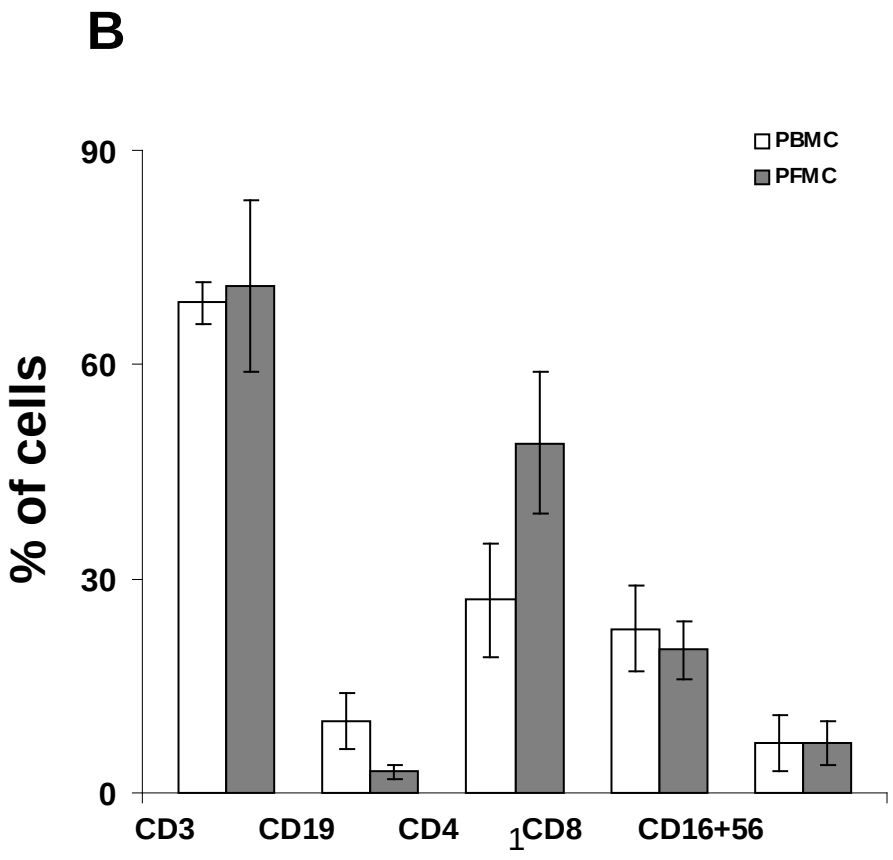
The rising prevalence of tuberculosis compounded by the increase in HIV infection and the emergence of drug-resistant strains of M. tuberculosis urge the need for better vaccines. The immune response against tuberculosis is known to be primarily cell mediated.



A. Cell Subset profile of 30 Tuberculosis patients

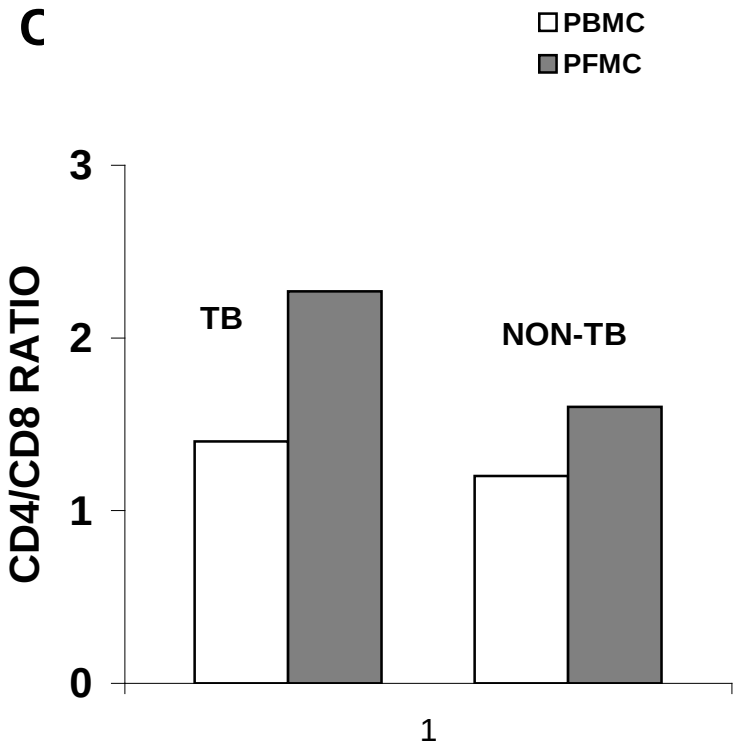
(Mean and S.E.M are represented in the graph;

Statistical significance is marked by *)



B. Cell Subset of 21 Non Tuberculosis Patients

(Mean levels and S.E.M are shown in the graph)



Ratio of CD4 to CD8 cells in TB and Non TB

Understanding the immune responses from a diverse group of patient population, especially the TB patients, would help us to understand the protective immune responses operating at local levels. To identify newer antigens that specifically elicit protective immune responses at a local site compared to that at the systemic level would be a substantial candidate for the development of new generation TB vaccines.

To start with, we investigated the in vivo correlates of immunity by studying the immunological architecture in PF and blood. Our flow cytometric data showed that there is an increase in the percentage of CD4 cells ($P < 0.05$) in PF as compared to that of blood in TB patients (Fig. A). Also there was an increase in the CD4/CD8 ratio in TB patients (Fig. C). A representative of these data acquired on flow cytometer is depicted in Fig. D.

The percentage of CD8 cytotoxic T cells in blood and PF of TB patients was higher than the control NTB cases (Fig. A and B). This marginal increase in percentage of CD8 T cells could be compensatory, in response to increased percentage of CD4 cells, a mechanism to maintain the CD4/CD8 ratio and an additional ‘acquired’ activation by these cells to control infection [12]. The distinct scenario of the CD4 T cells admixed with the CD8 cytotoxic T cells suggests that the lead role in CMI is played by the CD4 T cells. This is evident from the higher CD4/CD8 ratio seen with TB patients. The population of the CD16+56 NK cells showed a decrease at the local site of TB patients, indicating that the CD8 T cells might have a compensatory role to play in the cytotoxic killing of infected cells. The percentage of CD19 B cells was low in PFMC compared to PBMC, indicating a lack of enhanced B cell response in PF.

The higher CD4 cells in the PF and a greater CD4/CD8 ratio in TB patients is due to compartmentalisation, which is unique to TB and our results agree with it.

To evaluate further in vivo correlates of immunity at the site of infection, we measured the cytokine levels in the PF and in the BL that would reflect the in vivo helper T cell response in tuberculosis. Our results showed that there is selective concentration of IFN-gamma, TNF-alpha and IL-12 in the PF of TB patients (Fig. 3A), as reported by the previous studiesThis confirms that the TH1 type cytokine and hence TH1 type immune response occurs at the site of disease. The absence of any compartmentalised pattern of in vivo cytokine in NTB subjects (Fig. 3B) is because of the absence of mycobacteria or its antigens in the pleura unlike TB, where the T cells are more educated to mount an elective response to a second challenge and lead to compartmentalisation. It is known that IL-12 enhances IFN- production that has a protective role in tuberculosis [17]. In turn, IFN gamma stimulates TNF alpha secretion that brings about the pro inflammatory response in tuberculosis.

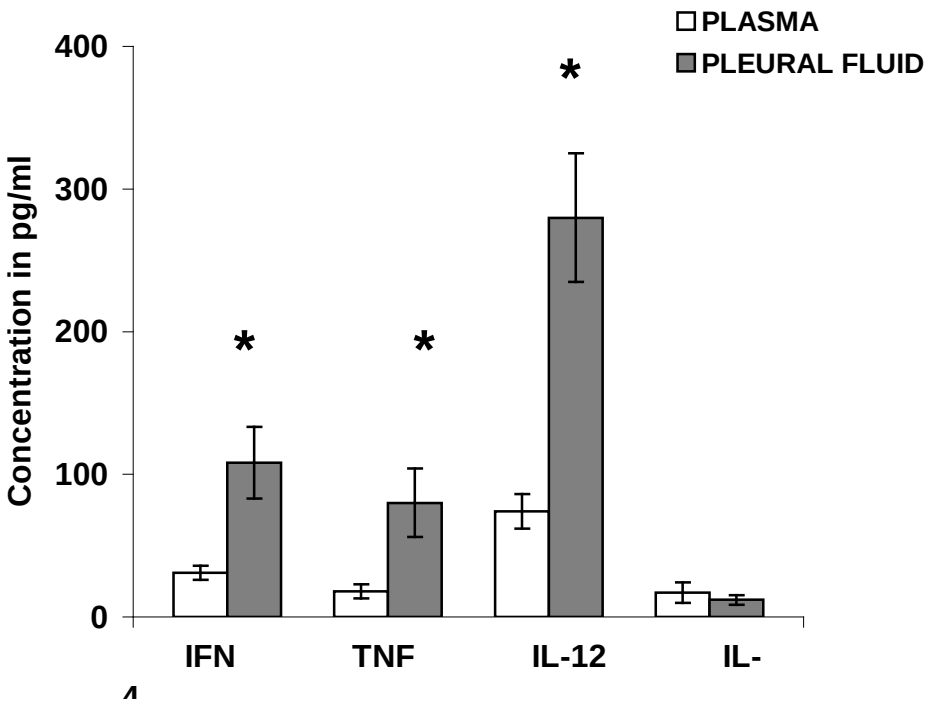


Figure E. Levels of IFNα, TNFγ, IL-12 and IL-4 in tuberculosis patients in Plasma and PF

(Mean and SEM are represented in the graph; Statistical significance is marked by *)

Together these cytokines are concentrated in the PF suggesting that these cytokines have important role in immunological resistance to tuberculosis, at the site of disease.

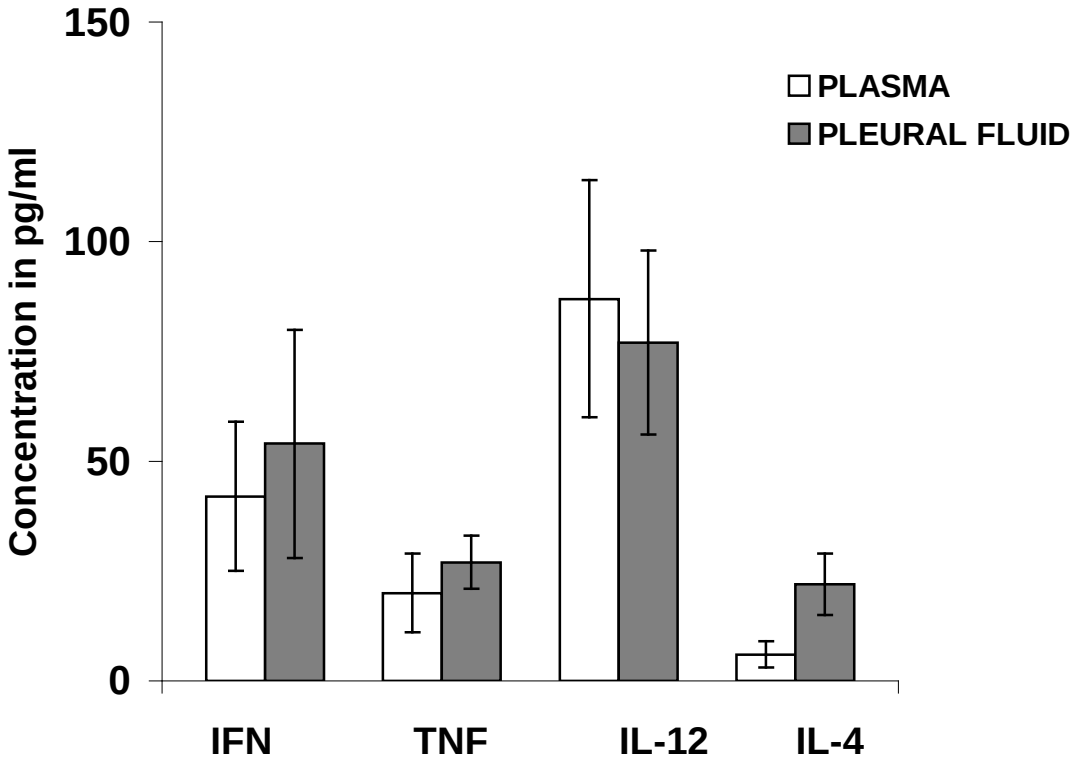


Figure F. Levels of IFNα, TNFγ, IL-12 and IL-4 in

Non tuberculous patients in Plasma and PF

(Mean and SEM are represented in the graph;

Statistical significance is marked by *)

On the other hand, IL-4, which is an anti-inflammatory cytokine that acts antagonistically to IFN- γ was decreased in PF of TB subjects. This decrease in IL-4 concentration may indirectly compel the need for the enhancement of protective TH1 immune response.

The in vitro cytokine response of the TB patients showed that the IFN-gamma levels increased in PFMC when compared with the PBMC in all stimulated conditions.

Interestingly, the IL-4 levels in all the stimulated conditions of PFMC were also significantly higher than the control levels (Fig. F). However, the IL-4 levels of PBMC in both stimulated and unstimulated conditions were almost similar and comparable to the PFMC control level and did not exhibit any prominent variation. This shows that the in vitro cytokine response shifts towards a TH0/TH1 type.

Thus, there is a differential T helper response in TB. This increase in IL-4 production in response to M. tuberculosis possibly influences the sensitization of T lymphocytes to apoptosis by a TNF alpha mediated pathway as suggested by Rook et al.

This study was able to delineate the correlates of protective immunity in TB at the local and at the peripheral level. Our results demonstrated that there is

selective TH1 response at the site of infection that is protective. This agrees with the previous studies concluding similar results [24-27]

However, further experiments have to be performed to show that T cells that are programmed to death are activated and to end the critical concentration of PPD that affects the T cell proliferation but not apoptosis and vice versa. These studies would help us to understand the antigen interaction with immunoreactive cells that would benefit protective response to host and thus form the basis for the vaccine construction.

Conclusion

1. In this study, we confirmed that cytokine status and T-cell reactivity within the tuberculous pleural effusions were polarized strongly toward a Th1 response.
2. Compartmentalisation of lymphocytes in pleural effusion occurs in the lymphocyte population in cases of tuberculous pleural effusion.

The future

Immunotherapy

This is perhaps the most important issue facing workers in the field of tuberculosis. As outlined earlier, DOTS helps, but fails to solve the problem, and Multidrug-resistant disease is an increasing threat. Immunotherapy is the only solution, and the need for this approach has been recognized since the time of R. Koch.

One potential approach to immunotherapy is the direct use of cytokines in patients, either systemically, or given by aerosol. IL-2, IFN γ, IL-12 and GM-CSF have all been investigated. Their potential roles in therapy, other than as a potential adjunct to drug treatment in multiresistant cases, have yet to be elucidated.

Another approach that gives striking results in mice is the use of DHEA or of the very similar androstenediol. These compounds oppose a subset of the effects of glucocorticoids, and can reverse the switch towards Type 2 cytokine profile in Balb/c mice, but this has not been tested in man.

Numerous works are being done in elucidating the unknown markers of mediators of apoptosis of Th1 cells in tuberculosis which is mediated by CD 95 cells. These research will throw more light on the immunology of tuberculosis and may be within a short period immunotherapy may play a pivotal role in tuberculosis.

Proforma

Name	:		
Age	:		
Sex	:		
Cough	:	Y/N	Duration
Evening rise of Temperature	:	Y/N	Duration
Loss of Appetite	:	Y/N	Duration
Loss of Weight	:	Y/N	Duration
BCG vaccination	:	Y/N	
Contact History	:	Y/N	
Diabetes Mellitus	:	Y/N	
HIV By ELISA	:	Y/N	
Steroids Use	:	Y/N	
Systemic Illness	:	Y/N	(If yes, in detail)

Pleural Fluid	:	
Biochemical	:	Sugar
		Protein
		LDH
Cytology	:	Cell Count
		Malignant cells
Microbiology	:	Gram Stain
		AFB Stain
		PCR
		Culture by BACTEC
FACS	:	CD3, CD4, CD8, CD16, CD56
Cytokines	:	INF γ, TNF α, IL-4, IL-12
Blood	:	CD3, CD4, CD8, CD16, CD56
	:	INF γ, TNF α, IL-4, IL-12

Master Charts

TB PB	AGE	SEX	IFN γ	TNF α	IL 12	IL 4	**CD3**	**CD19**	**CD4**	**CD8**	**CD16+56**	**CD4/CD8**
	42	M	35.8	32.7	79.4	28.7	44.72	4.65	43.06	23.37	40.54	1.84
	39	F	33.9	33.7	89.9	27	71.61	3.97	32.17	28.4	14.13	1.13
	55	M	48.8	35.1	109.3	18.8	74.82	15.21	30.83	33.76	11.62	0.91
	56	M	65.9	20.7	72.5	32.8	85.55	1.4	22.4	47.9	32.98	0.47
	52	F	38.2	22.9	84.9	21.5	61.46	1.29	28.9	57.41	18.94	0.50
	49	M	45.1	19.6	76.4	23.8	73.37	2.94	19.23	48.94	1.46	0.39
	62	M	35.2	45	78.9	27.7	79.76	9.16	14.62	2.89	19.7	5.06
	56	M	30.2	22.8	74	23.9	35.54	7.98	9.19	31.62	15.9	0.29
	51	F	39.6	20.5	75.9	23.4	63.9	5.5	24.5	34.3	11.6	0.71
	49	F	31.7	22.1	88.9	28.4	74.0	6.0	11.5	19.4	17.4	0.60
	42	M	35.4	22.5	81.9	26.5	73.9	10.3	28.4	39.9	19.4	0.71
	58	F	39.3	21.3	82.6	30.7	67.9	8.64	31.96	33.90	25.98	0.94
	62	M	34.2	25.4	88.2	24.8	87.18	7.98	28.97	38.72	6.94	0.75
	61	M	37.1	21.9	70.3	24.6	70.37	10.9	27.27	29.4	23.93	0.93
	59	M	32	19.6	73.7	21.3	60.86	11.8	28.64	31.74	12.36	0.90
	55	F	35.9	28.4	71.9	24.8	78.03	8.75	19.64	32.78	16.74	0.60
	59	M	26.9	24.5	70.3	25.8	55.97	9.4	38.53	38.27	10.47	1.01
	54	M	40.7	23.6	92.8	27.4	49.28	8.96	25.5	27.49	13.52	0.93
	64	M	35.9	25.4	82.9	24.9	56.91	10.68	29.7	30.28	12.6	0.98
	65	F	36.8	24.6	82.5	23.4	63.67	11.94	28.05	36.48	14.2	0.77
	57	M	45.9	23.7	89.3	25.4	61.58	9.73	32.05	38.91	8.6	0.82
	50	M	24.7	25.9	93.3	27.4	67.96	9.38	20.57	30.48	17.5	0.67
	53	F	41.8	24.9	80.2	24.6	63.72	9.85	22.63	30.42	14.2	0.74
	60	F	34.2	25.3	73.8	23.8	66.49	7.43	27.53	51.93	19.3	0.53
	49	F	36.8	23.5	72.9	24.7	63.26	6.9	29.74	38.49	13.6	0.77
	62	M	33.9	26.9	72.8	25.5	69.73	12.8	27.94	29.47	15.2	0.95
	58	M	32.1	29	60.2	24.6	75.38	11.5	26.9	20.38	12.76	1.32
	51	F	27	29.6	87.9	24.8	61.95	6.95	23.49	19.7	13.53	1.19
	53	M	63.8	26.6	83.9	30.4	58.05	8.5	22.84	14.8	19.39	1.54
	63	F	29.7	38	72.9	19.5	65.95	9.6	19.84	30.5	15.02	0.65
mean_1			37.62	26.19	80.48	25.36	66.09	8.34	25.89	32.40	16.32	0.99
SD_1			9.19	5.78	9.56	3.08	11.13	3.25	7.12	11.09	7.41	0.84
Z			-3.58	-4.10	5.71	1.17	-1.02	-1.39	-0.93	1.46	3.99	-0.41

TB PF	AGE	SEX	IFN γ	TNF α	IL 12	IL 4	**CD3**	**CD19**	**CD4**	**CD8**	**CD16+56**	**CD4/CD8**
	42	M	100.5	87.4	294.7	21.8	74.98	4.37	61.51	18.79	5.15	3.27
	39	F	98.7	89.4	305.8	23.8	82.38	2.58	58.82	25.94	7.45	2.27
	55	M	99.5	88.4	287.6	20.6	85.7	2.44	51.83	27	9	1.92
	56	M	130.8	84.9	294.9	18.3	83.27	1.65	54.19	21.38	4.03	2.53
	52	F	88.8	90.2	279.5	17.9	88.23	4.35	59.35	18.38	4.14	3.23
	49	M	104.5	110.3	296.9	21.8	81.85	8.47	72.73	11.16	2.25	6.52
	62	M	93.8	78.3	310.9	19.6	71.17	12.92	42.85	22.9	3.28	1.87
	56	M	94.3	75.9	280.5	21.4	87.13	1.95	73	24	2.83	3.04
	51	F	107.9	83.4	278.9	21.4	81.84	4.32	57.36	31.83	4.98	1.80
	49	F	108.7	87.9	293.4	23.8	79.39	5.93	59.92	29.17	5.38	2.05
	42	M	108.3	82.3	290.4	22.1	87.47	6.03	73.69	21.73	5.93	3.39
	58	F	96.7	80.4	291.8	18.3	74.73	7.47	72.48	19.35	4.37	3.75
	62	M	93.6	92.8	289.9	19.8	80.24	8.62	63.45	19.47	5.05	3.26
	61	M	104.6	87.4	290.4	18.4	87.35	10.84	54.37	26.67	3.49	2.04
	59	M	99.5	92.5	298.9	23.9	74.85	13.05	56.93	25.71	4.27	2.21
	55	F	95.7	93.4	289.7	20.5	72.29	9.73	51.93	20.39	5.39	2.55
	59	M	104.6	90.3	302.1	22.6	60.71	7.39	60.38	21.38	6.39	2.82
	54	M	103.5	95.5	294.8	22.3	63.89	5.29	52.75	23.27	5.05	2.27
	64	M	102.4	82.9	310.2	20.5	69.37	6.22	59.86	20.04	5.37	2.99
	65	F	104.7	98.5	304.8	22.4	72.37	8.41	64.79	16.37	4.38	3.96
	57	M	94.5	105.9	298.5	19.4	71.48	4.82	70.26	19.27	5.12	3.65
	50	M	94.7	94.8	305.6	20.4	72.39	5.93	73.49	15.48	4.83	4.75
	53	F	98.4	88.5	296.9	21.5	73.84	7.48	71.38	13.27	4.36	5.38
	60	F	105.6	93.9	298.5	22.9	74.26	3.73	70.82	15.47	5.27	4.58
	49	F	104.6	92.7	302.6	28.4	71.38	2.97	53.96	15.83	4.27	3.41
	62	M	106.4	93.8	301.8	17.4	73.83	3.85	52.73	23.28	5.25	2.27
	58	M	103.2	81.8	294.7	19.5	78.35	4.73	57.48	22.18	4.38	2.59
	51	F	93.5	93.4	294.8	16.9	83.17	5.38	62.52	18.37	4.16	3.40
	53	M	98.5	93.8	294.7	21.6	60.24	4.27	63.71	19.36	4.97	3.29
	63	F	100.5	78.9	295.7	18.5	58.73	3.59	62.83	21.39	5.38	2.94
Mean			101.37	89.65	295.6	20.92	75.90	5.96	61.38	20.96	4.87	3.13
SD			7.61	7.65	8.03	2.41	8.24	2.99	8.04	4.63	1.28	1.09
Z			29.20	31.98	132.8	13.08	2.59	-0.32	3.66	-3.90	-3.45	6.90

NTB PB	AGE	SEX	IFN γ	TNF α	IL 12	IL 4	**CD3**	**CD19**	**CD4**	**CD8**	**CD16+56**	**CD4/CD8**
	56	M	45.4	30	70	24	78.73	2.21	38.46	31.97	15.6	1.20
	36	F	42.9	32	69	23	61.2	20.6	16.55	21.25	1.2	0.78
	63	M	40.3	33	73	25	66.66	3.28	32.23	36.83	17.44	0.88
	49	M	48.9	31	72	28	60.83	12.05	2.1	1.58	0.96	1.33
	59	M	43.9	29	75	21	68.1	14	45.45	21.53	1.27	2.11
	63	M	40.4	28	68	22	67.32	10.428	26.958	22.632	7.294	1.19
	39	F	41.9	30	69	25	73.59	11.47	31.92	38.74	8.28	0.82
	53	M	46.2	32	68	28	70.03	9.38	29.37	28.39	10.27	1.03
	48	F	47.9	33	71	23	65.38	9.74	28.04	34.39	8.37	0.82
	53	M	51.8	35	72	21	62.37	9.39	19.38	31.28	12.46	0.62
	58	M	41.7	38	74	22	72.27	12.39	30.12	31.29	9.36	0.96
	59	M	42.4	31	69	26	63.36	7.49	29.26	29.47	7.38	0.99
	57	M	43.9	30	70	27	71.94	8	27.38	32.48	9.63	0.84
	54	F	41.3	33	69	23	70.27	6.87	21.78	38.41	10.49	0.57
	36	F	43.9	32	66	25	69.24	10.37	31.27	30.28	9.38	1.03
	58	M	42.6	35	73	21	72.85	12.25	30.28	23.38	10.37	1.30
	53	M	43.1	27	63	26	60.46	9.39	37.28	21.49	11.86	1.73
	48	M	41.8	29	65	28	68.58	9.74	28.94	28.49	10.38	1.02
	52	M	50.5	27	68	26	79.64	8.38	29.38	32.58	16.34	0.90
mean_2			44.25	31.32	69.68	24.42	68.57	9.86	28.22	28.23	9.39	1.06
SD_2			3.36	2.85	3.09	2.43	5.58	3.91	9.05	8.52	4.63	0.37

NTB PF	AGE	SEX	IFN γ	TNF α	IL 12	IL 4	**CD3**	**CD19**	**CD4**	**CD8**	**CD16+56**	**CD4/CD8**
	56	M	53.7	26.5	80.4	10.5	80.97	2.05	49.43	26.2	13.71	1.9
	36	F	55.9	29.6	78.9	9.7	65.84	4.39	52.59	27.8	8.96	
	63	M	59.5	23.6	73.5	12.5	88.29	3.05	54	29.81	7.69	1.8
	49	M	60.3	27.5	80.4	11.7	83.6	3.59	10.48	10.48	0.87	1.0
	59	M	52.9	30.5	84.5	13.6	32.88	4.75	18.39	14.42	11.52	1.3
	63	M	57.9	25.6	80.6	9.7	70.316	3.98	36.978	21.38	8.55	1.7
	39	F	51.5	21.6	83.4	7.9	67.33	6.35	76.7	35.59	9.47	2.2
	53	M	58.4	2.6	82.4	13.7	70.44	9.54	64.87	28.56	10.57	2.3
	48	F	53.7	31.5	75.8	15.7	45.98	6.59	56.49	28.39	8.26	2.0
	53	M	49.6	27.5	79.6	7.6	38.48	7.48	46.58	27.49	6.59	1.7
	58	M	51.6	28.6	79.6	9.5	53.8	5.5	34.3	30.2	8.7	1.1
	59	M	50.6	24.6	82.4	15.7	52.87	7.39	31.83	26.86	2.27	1.2
	57	M	55.3	23.6	83.5	12.7	35.67	18.39	48.49	28.48	8.49	1.7
	54	F	57.7	24.7	82.7	13.8	58.98	3.98	63.28	28.39	4.59	2.2
	36	F	55.8	28.5	80.6	9.6	75.78	5.94	48.38	28.49	5.93	1.7
	58	M	53.7	29.5	78.4	10.5	74.38	9.39	49.93	32.4	3.69	1.5
	53	M	57.4	27.4	83.7	11.6	67.94	3.47	45.87	34.89	7.63	1.3
	48	M	53.6	25.3	80.5	11.5	79.46	5.96	50.25	36.7	8.49	1.4
	52	M	48.6	25.4	84.6	13.6	85.85	7.49	52.83	40.94	6.95	1.3
Mean			54.62	25.48	80.82	11.64	64.68	6.28	46.93	28.29	7.52	1.63
SD			3.37	6.12	2.89	2.36	17.21	3.59	15.54	7.13	3.10	0.39

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